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PRINCIPAL INVESTIGATOR: Anton V. Cheltsov, Ph.D.

CONTRACTING ORGANIZATION: Scripps Research Institute
La Jolla, CA 92037

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6. AUTHOR(S)
Anton V. Cheltsov, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Scripps Research Institute
La Jolla, CA 92037

E-Mail: anton@scripps.edu

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Prostate cancer is the most common cancer in the United States and is the second leading cause of cancer death among men in most western countries. The activation of androgen receptor is a major factor contributing to progression of the prostate cancer. Therefore, the administration antiadrogens (androgen antagonists) is a vital part of currently used therapies. However, limited number of available antiandrogens, especially those against mutant forms of androgen receptor found in metastatic prostate cancer, affect successful outcome of a treatment. Major factor impeding discovery of novel antiandrogens was absence of crystal structure of androgen receptor in antagonist conformation. To tackle this issue, the model of the receptor in antagonist conformation was built and validated in vitro. The model was used to search for novel non steroidal antiandrogen ligands based on the structures of marketed drugs. Two ligands were identified as promising drug leads, inhibiting transcription of target genes at submicromolar concentration. One of the ligands was found to act mechanistically different from known androgen receptor antagonists.

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Introduction

Prostate cancer is the most common cancer in the United States and is the second leading cause of cancer death among men in western countries. The prostate is important for proper bladder control and normal sexual function in males. The male sex hormone testosterone (TST) (belongs to the group of androgen hormones) mainly controls the growth and working of prostate through the androgen receptor (AR) (1). The AR is a member of the steroid NR superfamily of ligand-dependent transcription factors (2). It mediates the effects of TST and dihydro-testosterone (DHT) in the cells. The binding of an *agonist* ligand induces AR to assume an *agonist* conformation that leads to transcription activation of target genes, or inhibition when AR is in *antagonist* conformation upon binding of an *antagonist* ligand. The ligand binding is the first step of AR activation. The following steps include dissociation of AR from heat shock proteins (HSP), dimerization and its transport from cytoplasm to nucleus (Fig. 1). Constitutive activation of AR is implicated in development and progressing of prostate cancer, especially of metastatic form (3-6). Thus, in addition of surgical removal of prostate gland, androgen ablation therapy is used, which is surgical or chemical castration combined with the administration of antiandrogens. The antiandrogens, also known as androgen antagonists, are compounds rendering androgen receptor inactive. Unfortunately, the number of clinically available antiandrogens, especially those against mutant forms of androgen receptor found in metastatic prostate cancer, is severely limited. Therefore the goal of a given research project is to discover novel antiandrogens against AR and its metastatic mutant forms by utilizing the most recent methods of computational biology.

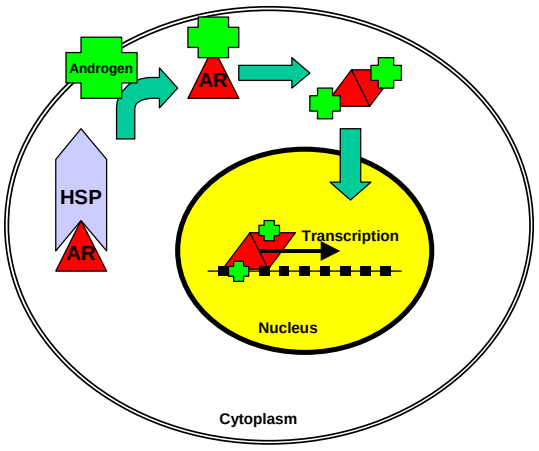


FIGURE 1. **Mechanism of action of the androgen receptor.** Upon binding of the ligand, AR frees from HSP, dimerizes, and then is translocated into nucleus, where it initiates transcription of target genes.

Body

Task 1.

Use protein structure modeling and docking technologies to understand the mechanism of agonist binding and specificity, and to design models of the antagonist bound conformations of AR and its metastatic mutant forms.

As of the day this report is being written, the Brookhaven PDB databank does not contain crystal structures for the AR ligand binding domain (LBD) in *antagonist* conformation. The figure 2 below displays crystal structures of homologous to androgen receptor, glucocorticoid receptor ligand binding domain in both agonist and antagonist conformations.

The current view of antagonism phenomenon is that more extended antagonist conformation of the nuclear receptor is unable to form transient complex with other nuclear factors, required for transcription initiation (7). The transition to antagonist conformation is mediated by the flexible loop (shown in green on Fig. 2) connecting helices 11 and 12, and by helix 12 (shown in yellow on Fig. 2). The flexible loop plays critical role by interacting with ligand. Thus, knowledge of the flexible loop structure in antagonist conformation is absolutely necessary for any virtual ligand screening experiments.

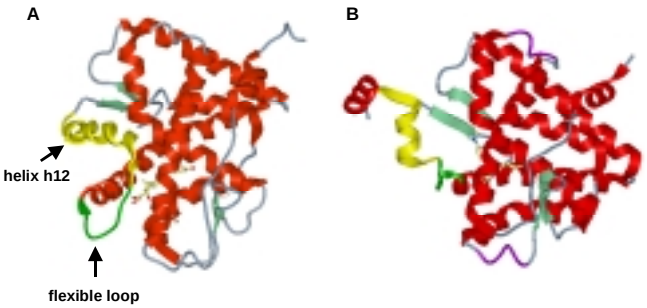


FIGURE 2. **Antagonism phenomenon in glucocorticoid receptor. A.** agonist conformation; **B.** antagonist conformation.

To address this question, comparative protein modeling was applied. Several structures of homologous nuclear receptors were tested as templates. However, docking of known antagonists and their derivatives produced mostly unacceptable results. The binding modes of ligands and their interactions with protein scaffold were not in accordance with published biochemical data. In particular, ligands did not form key interactions with residues, previously shown by site-directed mutagenesis to be essential for ligand binding (8-12).

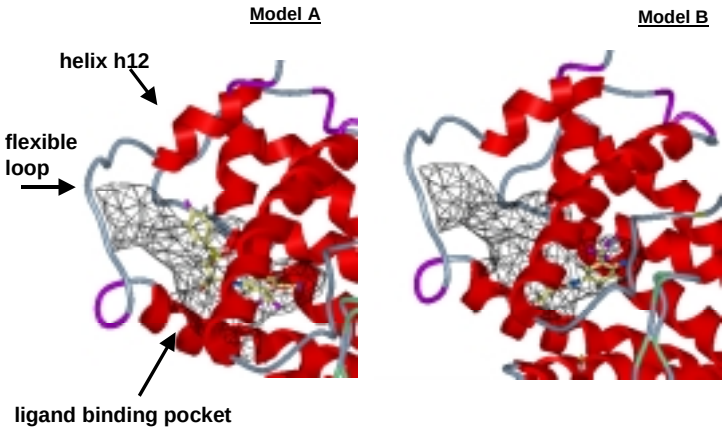


FIGURE 3. **Second generation models of AR LBD in antagonist conformation.** Ligand shown in stick-style are bicalutamide (Model A) and flutamide (Model B).

The analysis of homology models revealed that even though overall fold of all nuclear receptors LBDs is basically identical, the key residues of the androgen receptor LBD were placed into wrong secondary structure elements (e.g. into helix instead of loop and so on). These structural errors resulted mostly from poor sequence alignment due to numerous gaps. To tackle these issues, the best alignment, obtained with glucocorticoid receptor, was corrected manually, and threading-like procedure (as implemented in ICM) was utilized to generate initial approximation of the flexible loop and H12 conformations.

The initial model was further refined by iterative cycles of ligand docking, followed by resampling of side chains around docked ligands. The training ligand set consisted of flutamide, hydroxyflutamide, mifepristone and bicalutamide. This procedure was repeated iteratively until acceptable docking scores and ligand binding modes were achieved. From this set of models, two were selected for further development (Fig. 3).

As seen from the figure, the ligand binding pocket is of sufficient size (≈ 700 A^3) to be able to accommodate chemically diverse set of binders.

The training and refinement of the second generation models can be explained in the best way by the diagram (Fig. 4). The procedure was adopted with slight modifications from (13). Briefly, a small database (Database 1), containing published structures of 25 AR antagonists and their derivatives (14-18), was constructed. Each ligand from this database was docked independently into each of the models, generating 50 receptor-ligand complexes. Then, each complex was refined with ligand inside, followed by its redocking. Then, the database of 5000 compounds (Database 3), randomly selected from recent ChemDiv database, was docked to each of refined complexes, and docking score thresholds necessary to retain 1% and 10% of ligands were obtained. Then, the enriched database, containing 88 hand-picked agonists and antagonsists toward known nuclear receptors (Databse 2), was docked to each of the refined complexes. The docking results were converted into the enrichment factors, which reflect the ability of a particular model to select mostly nuclear receptor ligands (10% factor), and to select mostly AR ligands within this 10% pool (1% factor) (Fig. 5).

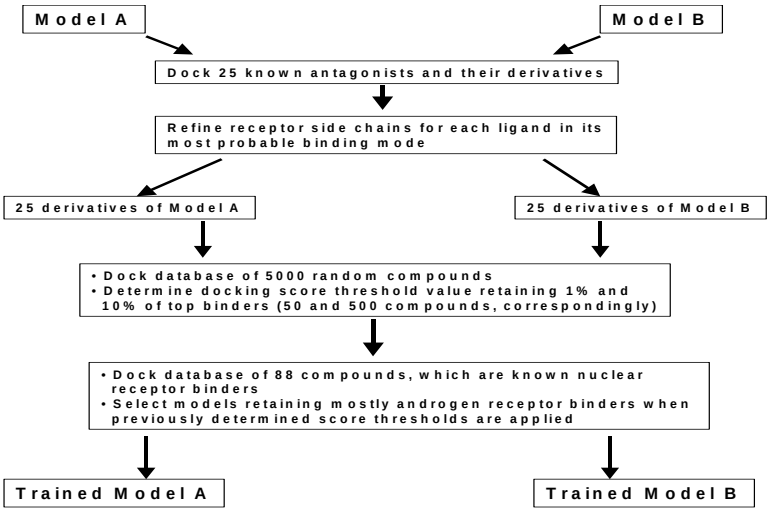


FIGURE 4. **Model training protocol.** Model A was refined with bicalutamide, and Model B with flutamide, respectively. 5000 compounds were randomly selected from ChemDiv database. Database of 88 compounds contained both agonists and antagonists for androgen, estrogen, glucorticoid, progesterone, retinoid, thyroid hormone, retinoic acid, pregnane X receptor and peroxisome proliferator activated receptor.

As seen from the figure 5, the best performing receptor LBD structures are model A derivative #5 (A5), and model B derivative #17 (B17). The models A5 and B17 were further tested for their ability to predict proper binding modes of known antagonists (Fig. 6).

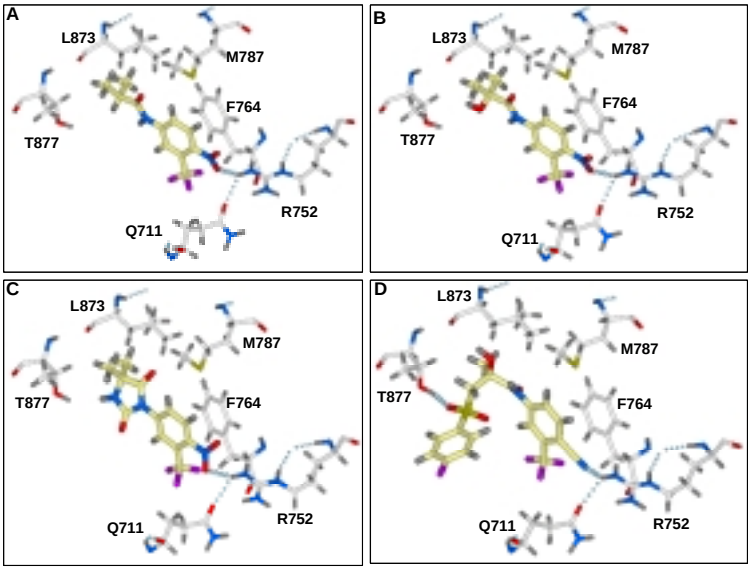


FIGURE 6. **Predicted ligand poses inside the A5 LBD model. A.** flutamide; **B.** hydroxyflutamide; **C.** nilutamide; **D.** bicalutamide. Hydrogen bonds are shown as dotted lines.

In particular, the formation of the hydrogen bond between ligand and the R752 residue was considered to be important. The R752 residue has been previously shown to be essential for ligand binding by numerous modeling and biochemical studies (19-23). As seen from the figure, all of the tested ligands were predicted to participate in hydrogen bonding with this residue. Similar results were obtained for the B17 receptor LBD model (data not shown). Based on obtained results, both A5 and B17 models were selected for virtual ligand screening (VLS) experiments and *in vitro* validation.

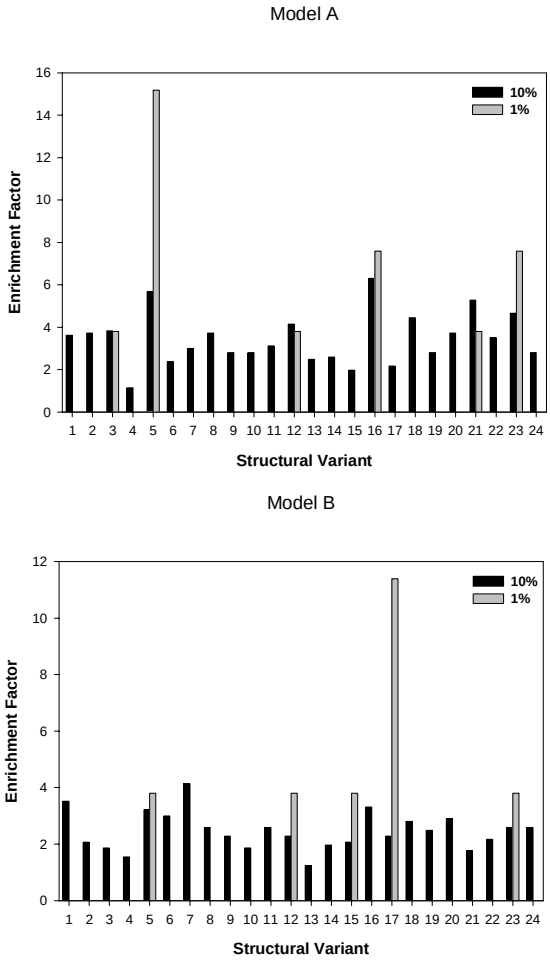


FIGURE 5. **Selection of best performing AR LBD models.** Grey and black bars represent enrichment factors value, corresponding to 1% and 10% cutoff, respectively. Structural variant number corresponds to compound index from Database 1, used to optimize the receptor side chains.

Task 2.

Identify potential antagonists by applying computational screening methods to developed AR LBD models.

Initial VLS experiments involved docking of the KEGG database (24) and the CNS library of ChemBridge. The top binders, scoring above 1% cutoff, were manually inspected and 16 compounds (8 from each model) were purchased. The transient transfection CAT assays were performed in collaboration with Prof. Xia Kun Zhang (The Burnham Research Institute), who has kindly provided his lab space, basic reagents and equipment for these experiments. The CAT experiments were performed as described in *Method* section of this proposal.

Most of the potential ligands did not exhibit either agonist or antagonist effects on the androgen receptor. However, one of the compounds (designated as #11) exhibited antagonist activity compared in magnitude to that of flutamide (Fig. 7).

In addition to CAT assays, competitive ligand binding studies have been also performed. These experiments have been done in collaboration with Prof. James T. Dalton (The Ohio State University), who kindly agreed to measure competitive binding K_I values with recombinant androgen receptor ligand binding domain (Table 1).The presented data do confirm that antagonist effects of #11 are due to its binding to AR LBD.

The literature search for ligand #11 revealed that it belongs to the phenothiazine (PhTZ) family of anti-

phycotic compounds, which are used in clinics to treat schizophrenia (25). These findings laid foundation for application of recent drug discovery strategy called *drug repurposing* (26). The drug repurposing is based on the concept that chemical space of drugs suitable for men rather limited. The biology and biochemistry of human body imposes rigid constraints on drugs structures, which must possess certain metabolic stability and bioavailability (27). There exist molecular motifs, which are associated with higher biological activity more frequently than other structures, and often confer activity on more than on target (28-31). As a result, the significant number of already approved marketed drugs possesses secondary activity due to interaction with the "off-targets". By derivatizing the original scaffold, one can enhance the desired secondary activity. The major benefit of this approach is the greater ease of introducing new compounds into clinics, since they "inherit" good bioavailability and toxicity profiles from the original structures.

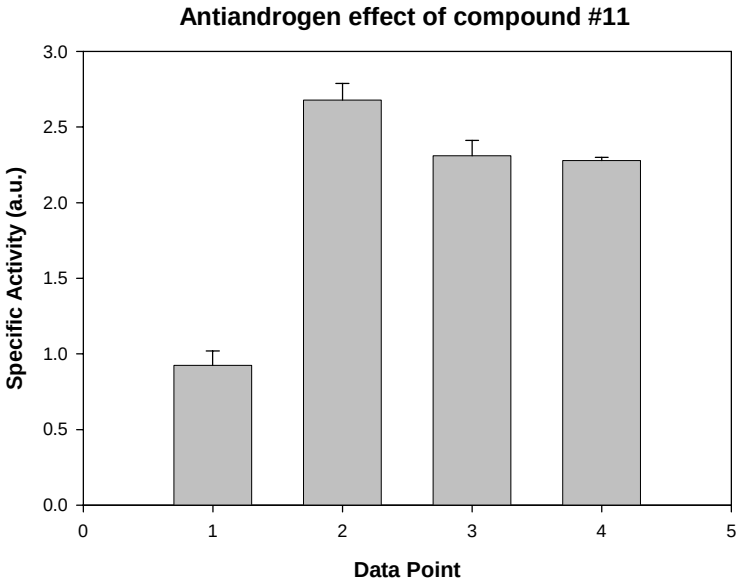


FIGURE 7. **CAT assay of compound #11.** Bars: 1 – control, 2 - 1.0 nM dehydrotestosterone (DHT), 3 – 1.0 nM DHT + 300.0 nM flutamide, 4 – 1.0 nM DHT + 300.0 nM compound #11.

TABLE 1. **Recombinant AR LBD competitive binding assay.**

Compound #	K_I, nM
1	n/a
2	n/a
4	n/a
5	n/a
6	n/a
7	n/a
8	> 1000.0
9	n/a
10	> 2000.0
11	850.0
12	n/a
13	n/a
14	n/a
15	n/a
16	n/a

n/a: no significantly competitive binding was observed;
#3: compound was skipped due to extremely poor solubility

The compounds, forming the intersection of best binders for both models, were then purchased (Table 2).

First, two best performing models were selected by docking ligand #11 into model sets A and B. Then, the database of available oral marketed drugs (27) was docked into the selected models. The results for one of such docking experiment are shown on the figure 8.

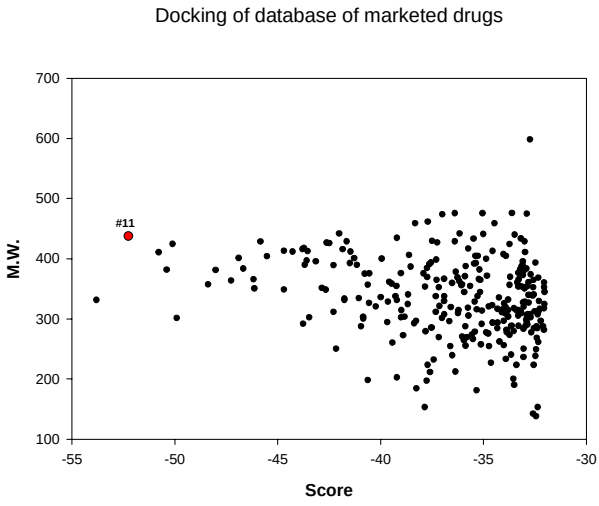
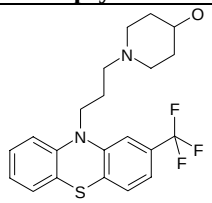
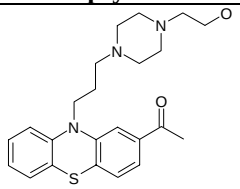
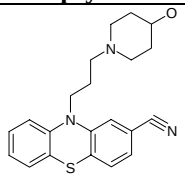
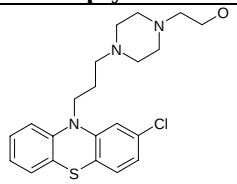
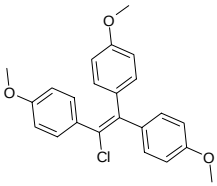
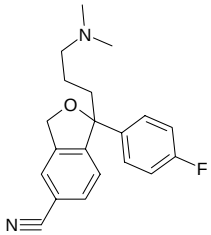
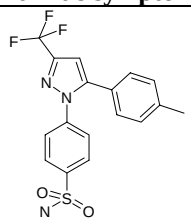
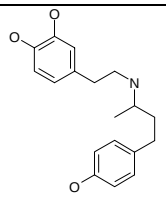
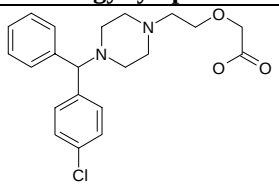
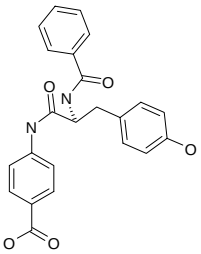
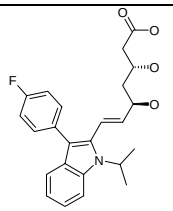


FIGURE 8. **Docking of marketed drugs database into the best scoring model.** Red circle is compound #11; more negative score corresponds to better ligand binding. Default ICM docking score cutoff of –32 was applied to data. Total of 1729 ligands docked. M.W. – molecular weight.

TABLE 2. **Selected marketed oral drugs listed with their name, pharmaceutical class and clinical application.**

Fluphenazine **Phenothiazine,** **antipsychotic**	**Acetophenazine** **Phenothiazine,** **antipsychotic**	**Periciazine** **Phenothiazine,** **antipsychotic**	**Perphenazine** **Phenothiazine,** **antipsychotic**
			
Chlorotrianisene **Estrogen,** **menopause treatment**	**Citalopram** **DHT reuptake inhibitor,** **antidepressive**	**Celecoxib** **Nonsteroidal AID,** **arthritis symptoms**	**Dobutamine** **Adrenergic β-agonist,** **cardiac anomalies**
			
Cetirizine **Antihistamine,** **Allergy symptoms**	**Bentiromide** **Diagnostic agent,** **pancreatic function**	**Fluvastatin** **reductase inhibitor,** **anticholesterol**	
			

Task 3.

Test the identified by VLS ligands for their antiandrogen properties using *in vitro* assays.

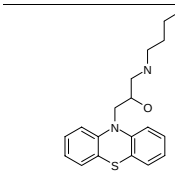
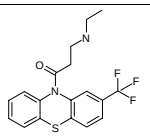
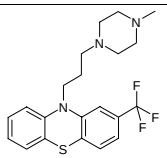
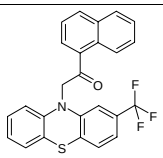
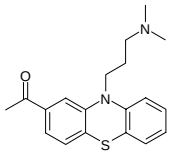
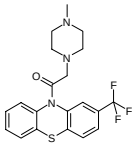
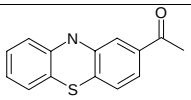
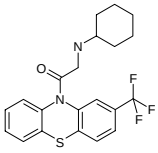
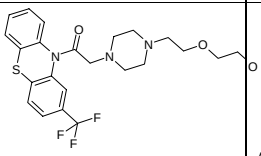
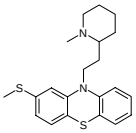
a) Wild Type AR Screening.

The compounds, shown in Table 2, were screened *in vitro* using transient transfection CAT assay, and three of the ligands were found to possess the desired antiandrogen activity (Fig. 9). The literature data have revealed that these drugs are dopaminergic antagonists, which target family of the G-protein-coupled receptors (32). During their administration, the male patients were reported to experience certain endocrinal side effects including loss of sexual desire and impotence (33). The observed side effects could now be explained by weak antiandrogen properties of these compounds, since the blood concentration achieved during administration of these drugs falls into micromolar range (34). These findings, together with the analysis of the predicted binding modes of the ligands, lead to the conclusion that the PhTZ system is the key structural determinant, conferring antiandrogen properties to the tested compounds. Thus, the PhTZ substructure search was performed in commercially available chemical databases in order to diversify the ligand set. Then, the selected ligands were purchased and screened *in vitro* using CAT assay (Table 3).

The ligands D4 and D12 were chosen for further studies. First, the effects of the ligand concentration on the antiandrogen properties were tested (Fig. 10). Next, the antagonism mechanism of the ligands was examined by performing AR nuclear localization experiments (Fig. 11). Briefly, the AR receptor was visualized in cells *in situ* by applying antibodies against AR and coupling them to secondary antibodies conjugated with fluorescent

markers. The antibody staining was performed with and without ligand treatment. The resulting AR distribution allows deducing the mechanism of action of a particular AR antagonist.

TABLE 3. **Antiandrogen activity of phenothiazine derivatives as determined by CAT assay.**

D1	D2	D3	D4
 AR(-) = 9%*	 AR(-) = 2%	 AR(-) = 3%	 AR(-) = 47%
D5	**D6**	**D7**	**D8**
 AR(-) = n/a	 AR(-) = n/a	 AR(-) = 12%	 AR(-) = n/a
D9	**D10**	**D11**	**D12**
 AR(-) = n/a	 AR(-) = 2%	PROPRIETARY DATA AR(-) = 34%	PROPRIETARY DATA AR(-) = 36%

* antiandrogen activity is expressed as degree of inhibition of the reporter gene transactivation by 1.0 nM DHT with 500 nM ligand.

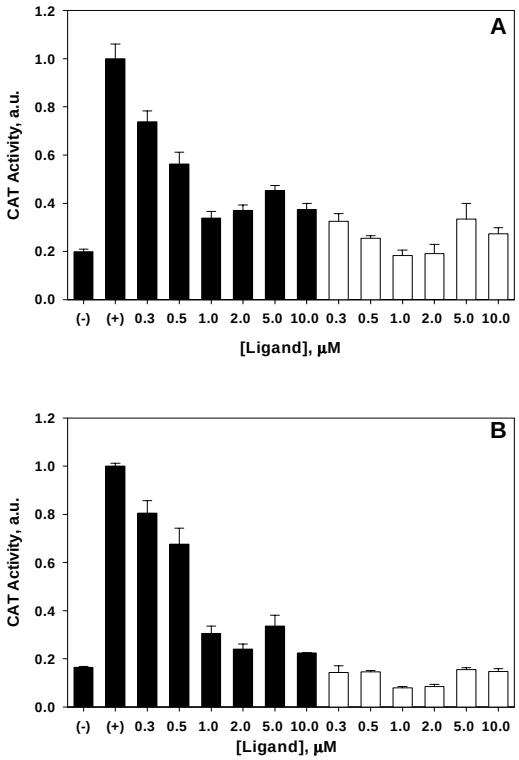


FIGURE 10. **CAT assays of the D4 ligand.** (-), untreated cells, negative control; (+) 1.0 nM DHT, positive control; filled bars represent experiments where 1.0 nM DHT was added together with the ligand at indicated concentration; white bars represent experiments where ligand alone was added at indicated concentration. **A.** D4; **B.** flutamide.

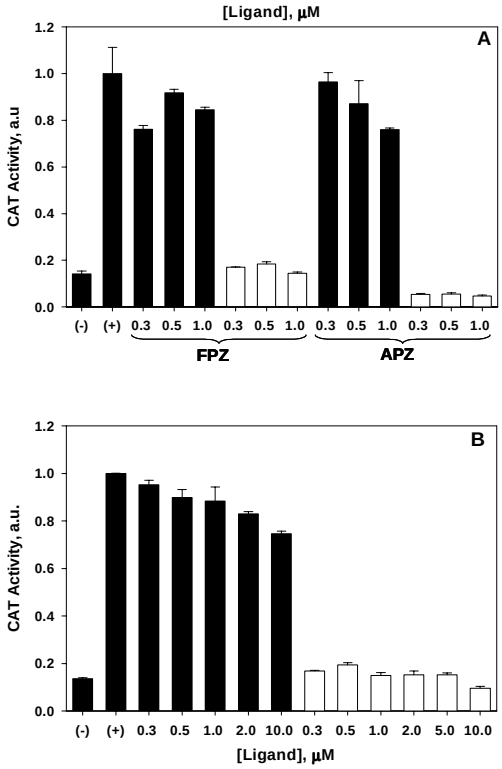


FIGURE 9. **CAT assays of selected marketed drugs.** (-) untreated cells, negative control; (+) 1.0 nM DHT, positive control; filled bars represent experiments where 1.0 nM DHT was added together with the ligand at indicated concentration; white bars represent experiments where ligand alone was added at indicated concentration. **A.** fluphenazine (FPZ) and acetophenazine (APZ); **B.** periciazine (PCZ).

The AR nuclear localization experiments have revealed that the mechanism of action of D4 is similar to that of classical antagonists such as hydroxyflutamide and bicalutamide. Accordingly, the D4 ligand binding seems to cause the AR to assume inactive antagonist conformation, which fails to initiate transcription of target genes upon translocation into nucleus. In contrast to D4, the D12 ligand achieves same effects in the opposite way. It blocks AR in the cell cytoplasm, even in the presence of huge excess of AR natural ligand DHT (K_I = 0.3 nM; [DHT] = 1.0 μM in the experiment).

Further experiments were designed to demonstrate the ability of the discovered ligands to inactivate endogenous AR. The metastatic prostate carcinoma cell line (LnCAP) was used for these experiments. These cells are known to be hypersensitive to androgens and to express high levels of prostate specific antigen (PSA). PSA is one of the AR activated targets, and a disease progression marker commonly used in clinics to assess a prostate cancer patient's condition (PSA assay).

The cellular PSA levels can be readily determined by immunoanalysis methods such as Western blot. The PSA assay was conducted for both ligands (Fig. 12). As seen from the figure, both D4 and D12 inhibited PSA expression in a dose-dependent fashion, without non-specific cellular toxicity (no change in α-tubulin

expression levels).

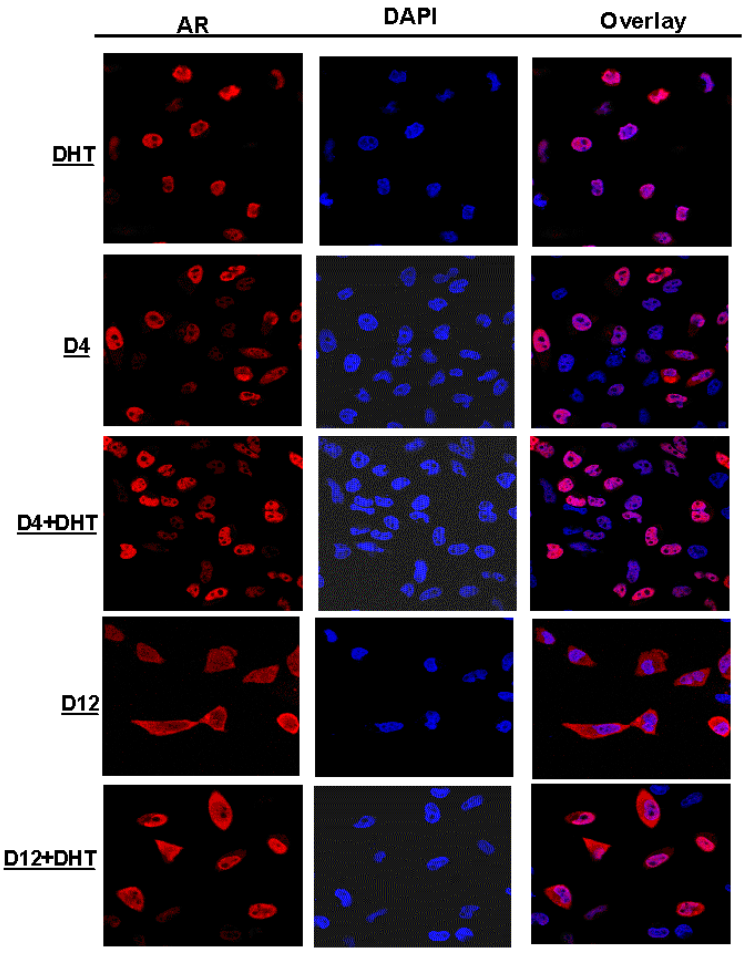


FIGURE 11. **AR nuclear localization experiments. AR**, immuno-staining with AR antibodies; **DAPI**, nuclei stained with 4,6-diamidino-2-phenylindole (DAPI); **Overlay**, digital superimposition of AR and DAPI columns. [Ligand] = 1.0 μM, [DHT] = 10.0 nM.

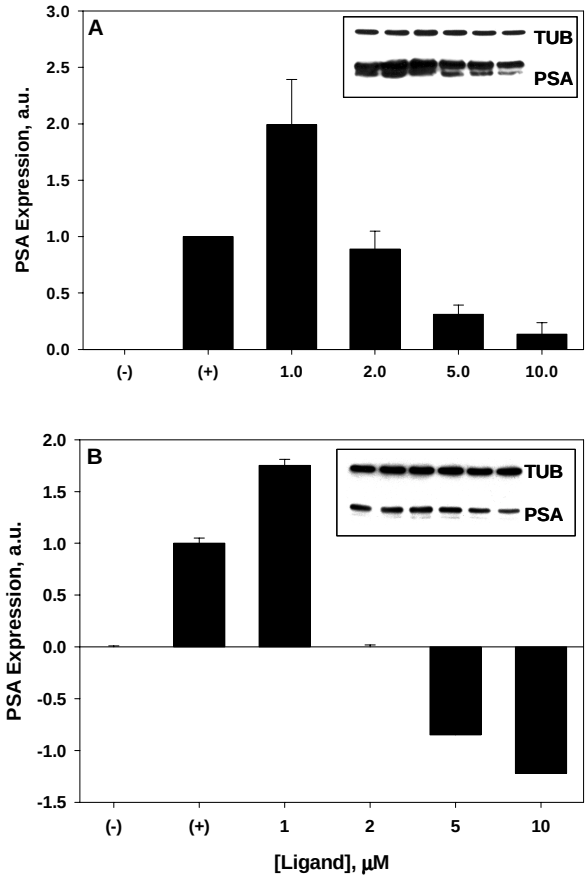


FIGURE 12. **The D4 and D12 ligands PSA assay. A.** D4; **B.** D12. **TUB**, α-tubulin; **PSA**, prostate specific antigen. (-), untreated cells, negative control; (+), 50.0 nM DHT alone, positive control. The ligand was added to the cells together with 50.0 nM DHT at indicated concentration. The films were digitized, and PSA expression data were normalized by α-tubulin content. **Inset:** typical Western blot.

The PSA assay demonstrates that the D12 ligand is much more potent antiandrogen than the D4. It achieved PSA expression inhibition below the basal PSA levels. These results agree with its ability to block AR in the cellular cytoplasm. Also note, that ratio [DHT]/[Ligand] in these experiments was 200, while it was 10,000 in CAT experiments.

The presented data convincibly demonstrate the power of employed drug discovery approaches. The D4 and D12 ligands, and associated structural scaffolds, are clearly promising drug leads. Both of the discovered ligands were derived from marketed drugs, and the main goal was to enhance the secondary activity of the original drugs, while eliminating primary. To test whether the derived from marketed drugs compounds still possess the original activity, their interaction with primary targets was investigated (Fig. 13). These experiments were conducted in collaboration with Prof. Patrick Sexton, Melbourne University, Australia. As seen from the figure, the D4 ligand does not possess significant activity toward studied receptors. The D12 compound does exhibit weak binding at milimolar concentrations, however it is not considered to be pharmaceutically important.

b) Metastatic AR Mutant Screening.

The antiandrogen properties of the D4 ligand were tested on the T877A AR mutant (most frequent metastatic mutation) at the Burnham Institute. Unfortunately, the D4 was found to behave as an *agonist* toward this mutant, activating AR target genes (data not shown).

In regard to the D12 ligand, the T877A mutant nuclear localization experiments are being conducted at the very moment this report is being written. The available wild type data suggest that this ligand might interact

with the AR in previously unobserved way. There exist several possibilities of such interaction, which include, but not limited to, the following:

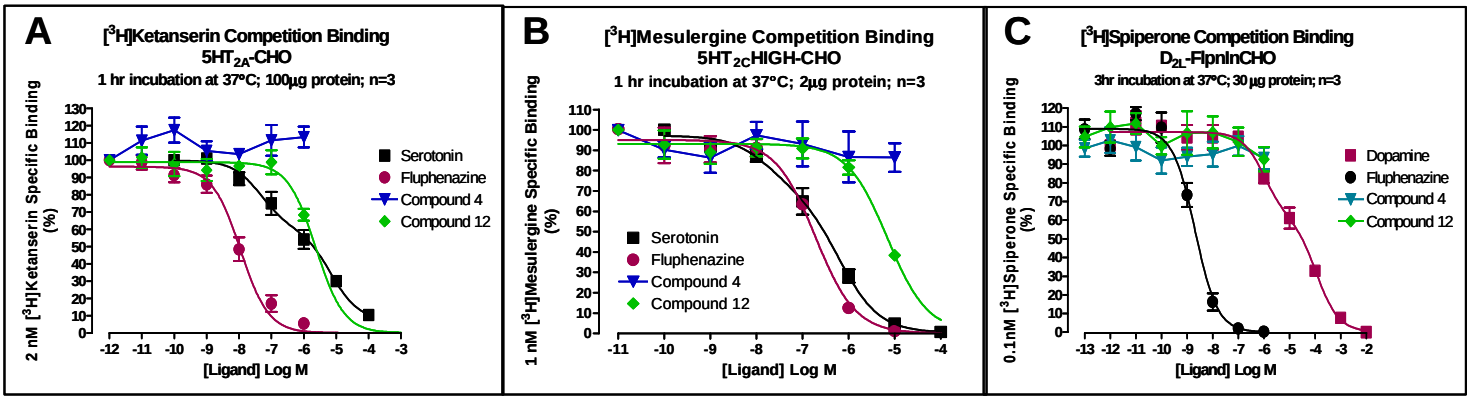


FIGURE 13. **D4 and D12 primary target activity assessment. A.** serotonin 5HT2A receptors; **B.** serotonin 5HT2C receptors; **C.** dopamine D2L receptors. Compound 4, D4; Compound 12, D12.

a) D12 interacts with AR LBD pocket, causing AR receptor to assume conformation, in which the binding pocket is no longer available for androgens, and AR is no longer able to dissociate from HSP;
b) D12 interacts with different part of AR the receptor, with an *exosite*, and causes similar situation as above;
c) D12 interacts with AR-HSP interface, stabilizing the cytosolic complex.

All three of the above scenarios will prevent AR from translocation into nucleus and from transcription initiation of the target genes. The scenarios b) and c) are beneficial in the way that the D12 might be able to *antagonize* both wild type AR and **ALL** of its known (and yet undiscovered) metastatic mutant forms. The properties of the D12 ligand are matter of ongoing investigation.

Key Research Accomplishments

- Model of the androgen receptor ligand binding domain in antagonist conformation has been built and biologically validated *in vitro*
- Novel non-steroidal antiandrogens against wild type AR LBD have been discovered
- One of the discovered antiandrogens has been shown to act on AR through previously unknown mechanism
- The new drug discovery methodology has been developed based on the drug repurposing approach and combination of computational and molecular biology techniques

Reportable Outcomes

1) The work has been presented as a poster at the 230th American Chemical Society Meeting & Exposition, August 28 - September 1, 2005 Washington, DC, USA. See appendix for abstract.
2) The resulting paper entitled "Discovery of Antiandrogen Activity of Non-steroidal Scaffolds of Marketed Drugs" has been submitted to the *Proceedings of the National Academy of Sciences*. See appendix for manuscript.
3) Another manuscript, dealing with the D12 ligand properties, is currently being prepared.

Conclusions

First, it has been demonstrated that the employed computational biology methodologies, combined with *in vitro* biological experiments, allow to surpass challenging drug discovery caveat imposed by the absence of the

wild type AR LBD crystal structure in the antagonist conformation. The model of the androgen receptor ligand binding domain in antagonist conformation has been built and validated *in vitro*.

Second, this model allowed to identify novel non-steroidal antiandrogen scaffolds, based on *in silico* screening of the database of FDA-approved marketed drug. The discovered scaffolds were further derivatized in order to enhance their secondary activity and suppress primary.

Third, the novel compounds are promising drug leads for further development due to their good cellular permeability and low toxicity, the qualities, they "inherited" from the original drug structures approved for use in humans.

Forth, the discovery of the D12 ligand opens new horizons toward the development of new generation of clinical AR antagonists.

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Appendices

ACS Meeting Abstract

IN SILICO VIRTUAL LIGAND SCREENING AS A POWERFUL TOOL TO DISCOVER SIDE EFFECTS OF MARKETED AND NOVEL DRUGS

W.H. Bisson[1] and A. Cheltsov [1], J. Chen[2], J.T. Dalton[2], X.-K. Zhang[3], and R. Abagyan[1]
[1]Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA, USA; [2]College of Pharmacy, The Ohio State University, Columbus, OH, USA; [3]Oncodevelopmental Biology, The Burnham Institute, La Jolla, CA, USA.

Widely used traditional drug discovery technologies such as high throughput screening, combined with combinatorial chemistry, and structure-based computational methods often do not perform well when applied to novel drug targets. Recently, it has been shown that by exploiting information about the known side effects of marketed drugs, it is possible to generate drug leads with enhanced selectivity toward the observed side-effect target and with superior bioavailability and toxicity profiles. This is the so-called "drug repurposing" approach. Here, we propose a methodology, which enables to discover leads, based on known drugs, to a given protein target. We demonstrate its application for discovery of novel non-steroid antagonists against human androgen receptor, using homology model as its structure. The homology modeling was combined with virtual ligand screening and full-atom receptor refinement to produce a set of models able to discriminate known androgen antagonists from other nuclear receptors binders and random false positives. The library of marketed drugs was then docked into best-performing models, and selected compounds were assayed *in vitro*. Three marketed drugs (AW 1-3), which belong to the same chemical family, were identified as mild androgen receptor antagonists. Their in vitro biological activity correlated well with endocrinal side effects observed in individuals taking these medications.

Classification: Biochemistry

Discovery of Antiandrogen Activity of Non-steroidal Scaffolds of Marketed Drugs

Bisson, W.H.[1*] and Cheltsov, A.V.[1*], N. Bruey-Sedano [2], X.-K. Zhang[2], and Abagyan, R.[1,¶]

[1]Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA, USA; [2]Oncodevelopmental Biology, The Burnham Institute, La Jolla, CA, USA; [*]both authors contributed equally to the work. [¶]Correspondence to: abagyan@scripps.edu.

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Abbreviations: AR, human androgen receptor; RARE, retinoic acid response element; HTS, high throughput screening; VLS, virtual ligand screening; LBD, ligand binding domain; DHT, dihydrotestosterone; BPMC, biased probability Monte Carlo; GR, glucocorticoid receptor; ER, estrogen receptor; CAT, chloramphenicol transferase; PSA, prostate specific antigen.

Abstract

Finding good drug leads *de novo* from large chemical libraries, real or virtual, is not an easy task. It suffers from inadequate diversity, low hit rates, and often produces leads that are toxic and/or exhibit poor bioavailability. Exploiting the secondary activity of marketed drugs, on the other hand, may help in generating drug leads with enhanced selectivity toward the observed side-effect target and with superior bioavailability and toxicity profiles. Here, we proposed and validated an efficient computational methodology to discover leads to a given protein target from safe marketed drugs. We applied this *in silico* "drug repurposing" procedure to identification of novel non-steroidal antagonists against human androgen receptor, using multiple predicted models of an antagonist-bound receptor. The library of marketed oral drugs was then docked into best-performing models, and only 16 selected compounds were tested *in vitro* using the transient transfection assay. The phenothiazine derivatives acetophenazine, fluphenazine and periciazine, used clinically as antipsychotics drugs, were identified as mild androgen receptor antagonists. Their *in vitro* biological activity correlated well with endocrinal side effects observed in individuals taking these medications. Further computational optimization of phenothiazines, combined with *in vitro* screening, led to novel non-steroidal anti-androgens with improved activity.

Introduction

Current approaches for discovery of novel chemical leads against a molecular target heavily rely on high throughput screening technology (HTS) and/or virtual ligand screening (VLS) techniques. The HTS technology proved to be useful for rapid lead identification for numerous drug targets (1-8). However, HTS was also found to have major drawbacks, which are significant level of false positives and false negatives, low hit rates, ubiquitous binding (9), poor bioavailability and toxicity profiles of lead compounds. These problems result partially from the nature of chemical libraries used for HTS. Furthermore, since the pharmacological properties of the screened compounds are largely unknown, there is a high risk that further optimization of hits identified with HTS will fail to evolve into real drugs.

In contrast, retrospective analysis of marketed drugs have shown that their physicochemical and structural properties seem to cluster around preferred values and scaffolds (10). It has been also observed that some chemical motifs are associated with high biological activity, and often confer activity against more than one target/receptor (11-16). These motifs have been referred to as "privileged structures" (11). These observations lead to an assumption that the chemical space of potential drugs is limited. Thus, the library of currently available drugs is a very attractive choice for finding suitable leads for further development and optimization for old or new targets/receptors. This library is small; the compounds in this library are drugs already, they do not need a long expensive optimization, and there is a high chance of finding a secondary activity which can be exploited.

In recent years, an increasing number of biotechnology companies have adopted this concept by focusing their research efforts on "drug repurposing", the development of novel uses for existing drugs. The drug repurposing consists of the identification of the secondary targets,

"off-targets", followed by the development of compounds designed to abolish the main activity and enhance the desired side effects. The major benefit of this approach is that once an existing drug is found to act on a novel target, its chemical scaffold can be used as a staring point for designing novel molecules with improved selectivity toward the new target, and good toxicity and bioavailability profiles "inherited" from the original structure. Thus, the repurposed compounds are likely to enter clinical trials more rapidly and at less cost, in contrast to new chemical entities derived "from scratch".

Successful examples of the drug repurposing include development from β-blockers potassium channel blocker levocromakalim (15), and development of an inhibitor of the fibrin transthyretine amyloid formation from non-steroidal antiinflammatory drug, flufenamic acid (16).

Here we describe and validate a computational method to identify secondary anti-androgen activity of known drugs. Recently, we have demonstrated that he ICM ligand docking and scoring approach with multiple models of nuclear receptors was able to enrich hit lists 33 to 100 fold for 9 out of 10 nuclear receptors studied (17) and predict specificity for 78 known nuclear receptor ligands reasonably well. We also demonstrated that a homology model can be used to identify new leads. We also identified novel antagonists to two nuclear receptors, retinoic acid receptor (18) and thyroid hormone receptor (19) using predicted antagonist-bound models.

The androgen receptor is critical for the development and progression of the prostate cancer (PC) and rather limited repertoire of clinically available antagonists exists. Here we developed multiple models for antagonist-bound conformations of the human androgen receptor, selected suitable models based on the known antagonists and applied these models for selecting

marketed drugs likely to have secondary anti-androgen activity. One of three families identified was confirmed by in vitro tests and further optimized.

Results

Model Optimization and Refinement. Figure 2 summarizes results of the implementation of the AR LBD model optimization and refinement protocol, as described in the *Methods* section. As the data suggest, higher values of 1% enrichment factor identifies models with stronger affinity toward AR binders. The difference between 1% and 10% enrichment factors, describes ability of a particular model to differentiate AR binders from other nuclear receptor binders. Accordingly, two structural variants were selected for VLS experiments from both model sets: A5 and B17.

Closer examination of variants A5 and B17 revealed RMSD difference of 0.8A within ligand binding site, resulting mainly from alternative packing of side chains. However, this small side chains rearrangement caused substantial differences in the volume and geometry of the ligand binding pockets, as predicted by the ICM pocket finder method (20). The volume of the A5 model pocket was found to be 689.0 A^3 with area of 650.0 A^2, while corresponding geometric features of the B17 pocket were 585.0 A^3 and 639.0 A^2, respectively.

Further comparison of selected structural variants involved redocking of Database 1, followed by manual inspection of docked ligands. While inspecting the ligand poses, particular attention was paid to interactions of ligands with amino acid residues, previously shown to be important for proper ligand binding by AR LBD (21-25). In particular, the formation of hydrogen bond between ligand and the R752 is speculated to be essential for proper ligand binding (Bisson, W.H., unpublished observations). This residue is highly conserved among known nuclear receptors, and recent crystallographic data demonstrate its importance for ligand binding for such receptors as estrogen and glucocorticoid receptors (26,27) as well as the W741L (28) and T877A (29) AR LBD mutants. Both the A5 and B17 structural variants were found to form this interaction with majority of the ligands from Database 1.

Virtual Ligand Screening. The marketed oral drugs database, described in (10), was used for ligand docking experiments. The database was docked into the A5 and B17 structural variants as described in *Methods* section, and the top binders with ICM docking scores ranging from –60.0 to –32.0 were examined manually. From the intersection of those, 11 compounds were selected and purchased for *in vitro* biological experiments (Table 1).

Transactivation and Binding Experiments. The summary of the results of the *in vitro* biological experiments is presented in Table 1. First, the ligands were tested for antagonism activity using CAT assay as described in *Methods* section. As the data suggest (Fig. 3A,B), compounds from the phenothiazine (PhTZ) family possess desired antiandrogen properties, with the exception of the perphenazine (Etrafon®) (PPZ), which did not demonstrate either antagonism or agonism toward AR.

Antiandrogen effects of fluphenazine (Prolixin®) (FPZ) and acetphenazine (Tindal®) (APZ) were not found to be dose-dependent at high ligand concentration. The cell treatment with periciazine (Neuleptil®) (PCZ), on the hand, exhibited dose-dependence (Fig. 3B).

The obtained transactivation data suggested that phenothiazines and their derivatives could be considered as a general scaffold for ligands, which might possess antiandrogen properties. Thus, changing the side groups of phenothiazines system should have effect on the antiandrogen properties of the ligands.

To test this assumption, the phenothiazine core substructure search was performed in available chemical databases, and a set of phenothiazine derivatives was purchased and screened *in vitro* (Table 2). The D4 ligand (Table 2) performed the best, inhibiting almost 50% of the reporter gene transactivation at 500 nM and 1.0 nM DHT in a dose dependent fashion (Fig. 4A). This level of inhibition is similar to flutamide , a well established anti-androgen (Fig. 4B).

Nuclear Translocation and PSA Assay Experiments. The cellular localization in response to ligands was also investigated (Fig. 5). In the absence of its natural ligand (DHT), AR is diffusely distributed in the cytoplasm. Upon treatment with 10.0 nM DHT for 1 hour, AR was completely translocated into the nucleus. The identical AR distribution was observed when cells were treated with the D4 ligand at 1.0 μM both in the presence and absence of DHT. The D4 ligand caused 90% of AR to translocate into nucleus.

The effects of the D4 ligand on the endogenous PSA expression in LNCaP cells were investigated (Fig. 6). At 5.0 μM the D4 ligand caused 70% inhibition of the PSA expression if compared to that of the positive control. Also note, that maximum [ligand]/[DHT] ratio in this experiment is 200, while it is 10000 in the CAT experiments.

Discussion

Absence of the crystal structure of the AR LBD in an antagonist-bound conformation makes receptor structure based virtual compound screening rather challenging. The chemical diversity of known AR binders suggests that the AR ligand binding site is capable of substantial induced flexibility to accommodate a binder. Previously we have generated productive antagonist-bound models for two nuclear receptors: the thyroid hormone receptor (19) and the retinoic acid receptor alpha (30). The available structures of antagonist conformations of the homologous nuclear receptors demonstrate that the loop connecting helices H11 and H12 is the most flexible element of their binding site. Therefore, the backbone flexibility of the corresponding AR loop should be taken into account during modeling in either implicit or explicit way.

The backbone flexibility was addressed during optimization of initial AR LBD models A and B. Analysis of these models has shown that RMSD difference between them (≈ 1.0 A) was contributed mainly by side chains atoms, while backbone atoms contribution was found to be insignificant (< 0.1 A). Therefore, the generation of series of derivative models (A1-24, B1-24) involved side chain optimization only. Also, it should be realized that the designed optimization protocol favors models, which are able to discriminate AR antagonists from AR agonists solely on the basis of the ICM docking score. Thus, the optimized receptor models might incorporate certain virtuality of the structure. As a result, the high docking score might suggest that a given ligand has a good chance to be a binder, but its predicted binding mode does not necessarily reflect the reality.

The validation of the best performing models involved docking of the known AR antagonists such as flutamide, hydroxyflutamide, nilutamide and bicalutamide. All of them were

found to from strong hydrogen bond with the R752 residue, located deep in the AR LBD binding pocket (data not shown). We speculated that this interaction is essential for proper binding of AR ligands, which secures them in the pocket. Indeed, this interaction was observed in the crystal structure of the hAR LBD in complex with R1881, where 3-keto group of R1881 is involved in formation of hydrogen bond (31), and in the crystal structures of AR LBD mutants W741L (28) and T877A(29) in complex with bicalutamide and hydroxyflutamide, respectively. Yet another example was the hydrogen bond between the 4-nitro group of hydroxyflutamide and R752 in the homology model of the AR LBD (32), (Bisson, W.H., unpublished observation). Lastly, the point mutation R752Q of the residue has been shown to be the reason for the X-linked syndrome of androgen insensitivity, also known as testicular feminization (*Tfm*) (33). The pathology of the disease results from a failure of tissue to respond to androgen. The interesting feature of this AR mutant is that while demonstrating modest decrease in affinity to R1881 if compared to wild type AR (K_I(R752Q)=1.2 nM *vs.* K_I(WT)=0.64 nM), the dissociation half-time of the same ligand is dramatically diminished ($t_{1/2}$(R752Q)=12.0 min *vs.* $t_{1/2}$(WT)=150.0 min)(34). As a result of this rapid ligand dissociation kinetics, the R752Q mutant requires at least 10,000 fold higher DHT concentration than wild type AR to achieve comparable transcription activation (34).

When the database of marketed oral drugs was docked into validated AR LBD models, the phenothiazine family of anti-psychotic compounds was found among the top predicted binders. The docking experiments generated an array of conformations of similar energy, but with rather different orientations. In particular, the hydrogen bond with the R752 residue was predicted to be frequently formed by either the substituent at the position C2 of the PhTZ system or by the hydroxyl of the 1-piperazineethanol group, which is common for these compounds.

This observation is in a good agreement with the statements, describing importance of the ligand interaction with the R752.

Three of the PhTZ derivatives (FPZ, APZ and PCZ, see Table 2) exhibited modest binding and antiandrogen activity *in vitro*. The literature search revealed that these drugs are routinely used in clinics to treat schizophrenia (35). They are dopaminergic antagonists, which target family of the G-protein-coupled dopamine receptors (36). During their administration, the male patients were reported to experience certain endocrinal side effects including loss of sexual desire and impotence (37). The observed side effects could now be explained by weak antiandrogen properties of these compounds discovered here, since the blood concentration achieved during administration of these drugs falls into micromolar range (38), and maximum *in vitro* AR antagonist activity of the FPZ and APZ was observed at 300 nM ÷ 1000 nM ligand concentration range (Fig. 3A).

The antiandrogen activity of these drugs could be attributed to the PhTZ system, which is topologically similar to the rigid steroid scaffold. The hydrogen bond acceptor group at the position C2 mimics groups of similar nature at the equivalent position of the steroidal ligands (e.g. 3-keto of the R1881 and DHT). This feature appears to be important for these scaffolds, since PPZ, which differs from FPZ only by chlorine substitute at C2, did not exhibit any AR related activity. Thus, it seems plausible that the preferred mode of binding for these compounds would involve formation of the hydrogen bond between C2 substitute and the R752, while the side chain at position N10 would interact with H11-H12 loop and H12 helix. This binding mode is similar to those observed for antagonists for other nuclear receptors (27,39). The antiandrogen properties of the D7 and D4 ligands seem to corroborate this assumption. The ability of the D4 ligand to induce translocation of the AR from cytoplasm into nucleus also suggests that its

antiandrogen activity is mechanistically similar to that of flutamide and bicalutamide (40). Another question, whether these derivatives still possess the original activity of the parent compound, needs to be addressed in the future experiments.

The structural diversity of the discovered antiandrogens provides further evidence that AR LBD possesses great flexibility to accommodate ligands of varied size and chemical nature. One of the reasons for such binding "unscrupulousity" could be a requirement for dependence of the transcription regulation on the cellular or tissue type context and other transcription activation mechanisms involved. The possibility of crosstalk among different transcription regulation pathways, when the same ligand might activate one pathway and down regulate the other, should not be discarded either.

Conclusions

In the present work, we have successfully demonstrated application of the "drug repurposing" approach to identify novel anti-androgen scaffolds. The compounds derived from a drug, marketed for another indication, possessed sub-micromolar anti-androgen activity and became promising leads for further optimization. The methodology developed in this work as well as the identified chemical scaffolds is currently being applied to discovery of novel antiandrogen lead candidates against metastatic mutant forms of the AR.

Materials and Methods

Model Preparation and Optimization. The initial approximation of antagonist-bound conformation of the AR ligand binding domain (LBD) was derived by combining methods of comparative protein modeling with global energy optimization. Briefly, the AR LBD structure for residues 669-885 was modeled using as template the crystal structure of the glucocorticoid receptor (GR) in antagonist conformation, bound to RU-486 (mifepristone) (pdb# 1nhz, (27)). To model the conformation of the loop, connecting helices H11 and H12, and the H12 helix (residues 886-910), different approach was employed. First, the weighed harmonic restraints were imposed between C_a of the residues 892-910 (H12) of AR LBD and C_a of equivalent residues of H12 (535-547) of the estrogen receptor (ER) in antagonist conformation (pdb# 1err, (41)). Then, φ, ψ and χ angles of the residues comprising the AR LBD flexible loop (residues 886-891) were freed and the energy of the system was globally optimized in the internal coordinate space using the Biased Probability Monte Carlo (BPMC) procedure (42,43) with the ICM program. The initial crude model was used to dock a small set of known agonists and antagonists, composed of testosterone, dihydrotestosterone (DHT), RU-486, flutamide, hydroxiflutamide, nilutamide and bicalutamide. Each receptor-ligand complex was then refined by BPMC procedure applied to flexible loop backbone and receptor side chains in the vicinity of the ligand, while allowing the ligand to move. The set of ligand was then re-docked to each of the refined receptor conformations. The resulting receptor-ligand complexes were manually inspected, and several receptor conformations, achieving best docking score separation between antagonists and agonists were chosen. The selected conformations were then used to generate a new set of refined receptor-ligand complexes, and the procedure was repeated iteratively to achieve a reproducible agonist/antagonist separation. The described procedure resulted in the

generation of two receptor conformations favoring antagonist molecules in terms of the ICM docking score. These models were designated as Model A (bicalutamide ligand scoring highest) and Model B (flutamide ligand scoring highest) and selected for further optimization and refinement. The optimization of selected receptor conformations is described by the following diagram (Fig. 1). Briefly, a small database (Database 1, see *Databases Preparation* section), containing published structures of 24 AR antagonists and their derivatives, was constructed. Each ligand from this database was docked independently into each of the models, generating 48 receptor-ligand complexes. Database 1 was docked at least three times, and the best energy ligand conformations retained. Then, for each receptor-ligand complex, conformations of the receptor side chains in the vicinity of the ligand were optimized by global energy minimization with BPMC in internal coordinate space (43,44). Then, the enrichment factors were computed for each refined model as described before (17), by determining docking score thresholds necessary to retain 1% and 10% of the top scoring ligands of the source database. The source database (Database 3, see *Databases Preparation* section), consisting of 5000 compounds, was docked to each of refined complexes three times. Then, a focused database, containing 88 agonists and antagonsists of known nuclear receptors (Database 2, see *Databases Preparation* section), was docked to each of the refined complexes. The enrichment factor corresponding to 1% docking score cutoff was computed by counting focused library hits only for AR ligands, while the enrichment factor, corresponding to 10% cutoff, was computed for the whole focused library.

Virtual Ligand Screening. The ICM virtual library screening (VLS) method was used as before (17). The portion of receptor in vicinity of 8.0A of the binding site was selected. Five grid potential maps, representing receptor selection, were generated. These maps accounted for the

hydrophobic, heavy atom and hydrogen van der Waals interactions, hydrogen-bonding interactions and electrostatic potential. Ligand molecules were prepared for docking by energy minimization in the absence of the receptor, and the lowest energy conformations were used as starting points for simulations of docking to receptor potential maps by the ICM method (45,46). The quality of ligand pose prediction was evaluated by assigning the score, generated by the ICM scoring function (47). Since the ICM docking method has a stochastic element, the docking simulations were conducted three times to ensure convergence. The ligand conformations with the lowest (best) scores were retained.

Database Preparation. Three databases (1, 2 and 3) were constructed and used to evaluate and optimize the AR LBD models. Database 1 contained 24 AR antagonists taken from literature (flutamide, hydroxyflutamide, nilutamide and derivatives, bicalutamide and derivatives, isoxazolone derivatives) (48-52). Database 2, containing 88 agonists and antagonists specific towards androgen, estrogen, progesterone and other homologous nuclear receptors (17), was kindly provided by Dr. M. Totrov from Molsoft, LLC. Finally, database 3 was generated by randomly selecting 5000 compounds from the ChemBridge drug-like compound database.

Plasmid Preparation. Expression vectors for full size AR, reporter gene *β*RARE-*tk*-chloramphenicol transferase (CAT) and *β-gal* expression vector (pCH 110, Pharmacia) were prepared from transformed *E.Coli* bacteria using QIAprep plasmid purification kit (QIAGEN).

Receptor Transcriptional Activation in Cotransfected Cells. Expression vectors for AR, CAT and *β-gal* were prepared as above. CV1-cells were routinely maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% of streptomycin/penicillin. For transfection assay, cells were seeded at 1.0×10^5 cells/mL in 24-well plates for 16-24h before transfection. Cells in each of the 21 wells (3 wells were kept with raw

cells) were then transfected using the FUGENE reagent (Roche) with 35 ng of AR, 300 ng of CAT and 100 ng of a *β-gal* per plate well. Following the transfection, cells were incubated for 4 hours, then ligands were added, and cells were allowed to grow for 24 hours. Following 24 hour incubation, cells were washed with HBS buffer and disrupted in 200 µl of 0.25M Tris-HCl buffer (pH 7.8) by freeze-thaw cycles. CAT activity was expressed relative to *β-gal* activity to normalize for transfection efficiency. All experiments were performed in triplicate.

Nuclear Translocation Experiments. Nuclear translocation experiments were performed in HeLa cells, which were routinely maintained in DMEM medium, supplemented with 10% charcoal treated (FBS). For translocation experiments, cell were transfected with 35 ng AR pcDNA3 (ref) using FUGENE reagent (Roche) and allowed to grow for 24 hours. Then, ligands were added to the cells to a final concentration of 1.0 µM, and cells were incubated for 2 hours. When both DHT and a ligand were used for treatment, ligand was added first, and 30 min later, DHT to a final concentration of 10.0 nM. Following incubation, cells were briefly washed with PBS and fixed with PBS containing 3% Formaldehyde and 2% sucrose for 25 min at room temperature, and then permeabilized with PBS containing 0.4% Triton X-100 and 5% goat serum for 20 min at room temperature. The fixed cells were first incubated with polyclonal anti-AR (Santacruz) for 2 h at room temperature, washed with 0.1% Triton X-100 in PBS, and then incubated with anti-rabbit antibodies (Alexa, 594) for 1h at room temperature. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for 15 min and washed three times with PBS. To observe cellular AR distribution, the cells were mounted on glass slides with VectaShield (Vector Laboratories). Confocal images were acquired by using a Zeiss Laser Scanning Microscope and analyzed with LSM510 software.

Prostate Specific Antigen (PSA) Assay in LNCaP Cells. The human androgen responsive prostate carcinoma cell line (LNCaP) was obtained from ATCC. The cells were routinely maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin. For PSA assay, cells were seeded at 1.0×10^5 cells/mL in 6-well plates and allowed to grow for 24 hours. Then, medium was replaced by basic RPMI and ligands were added. Following 48 hour incubation, the LNCaP PSA expression levels were assessed by Western blot analysis as previously described (53). To account for uneven protein loading, the PSA expression levels were normalized by α-tubulin content. The PSA polyclonal rabbit antibodies were purchased from DakoCytomation, and monoclonal mouse α-tubulin antibodies were obtained from Sigma.

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Figure Legends

FIGURE 1. **Model optimization and refinement protocol.** Model A scored best with flutamide, and model B with bicalutamide, respectively. Database 1 contained certain AR antagonists and their derivatives. Database 2 contained mixture of 88 agonists and antagonists specific toward androgen, estrogen, progesterone and other homologous nuclear receptors. Database 3 was composed from 5000 compounds randomly chosen from ChemBridge database. See text for details.

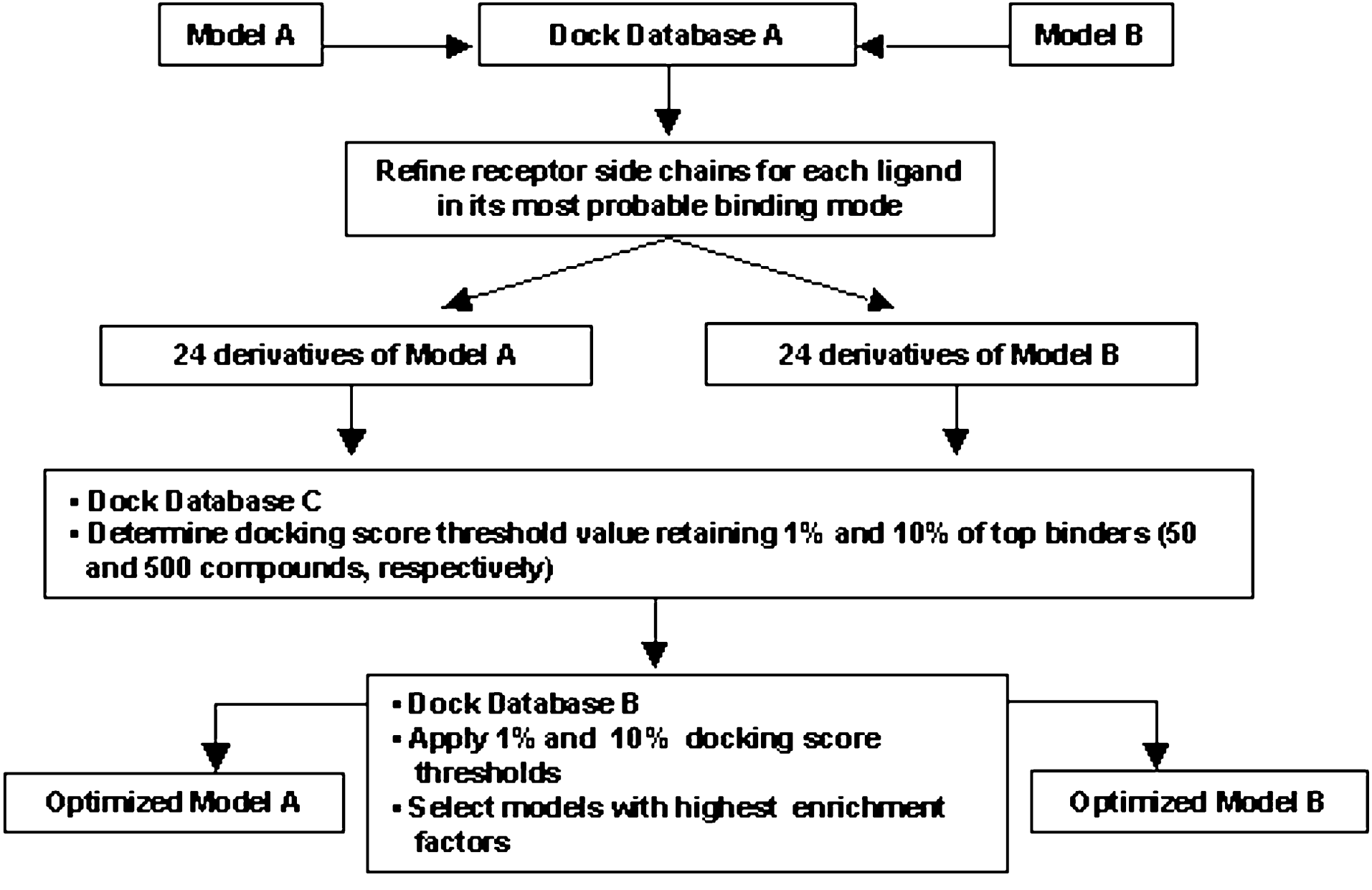
FIGURE 2. **Selection of best performing AR LBD models.** Grey and black bars represent enrichment factors values, corresponding to 1% and 10% cutoff values, respectively. Structural variant number corresponds to compound index from Database 1, used to optimize the receptor side chains. Enrichment factors were computed as described in the *Methods* section.

FIGURE 3. **CAT assays of selected marketed drugs.** (-), untreated cells, negative control; (+) 1.0 nM DHT, positive control; filled bars represent experiments where 1.0 nM DHT was added together with ligand at indicated concentration; white bars represent experiments where ligand alone was added at indicated concentration. **A.** fluphenazine (FPZ) and acetophenazine, (APZ); **B.** periciazine (PCZ).

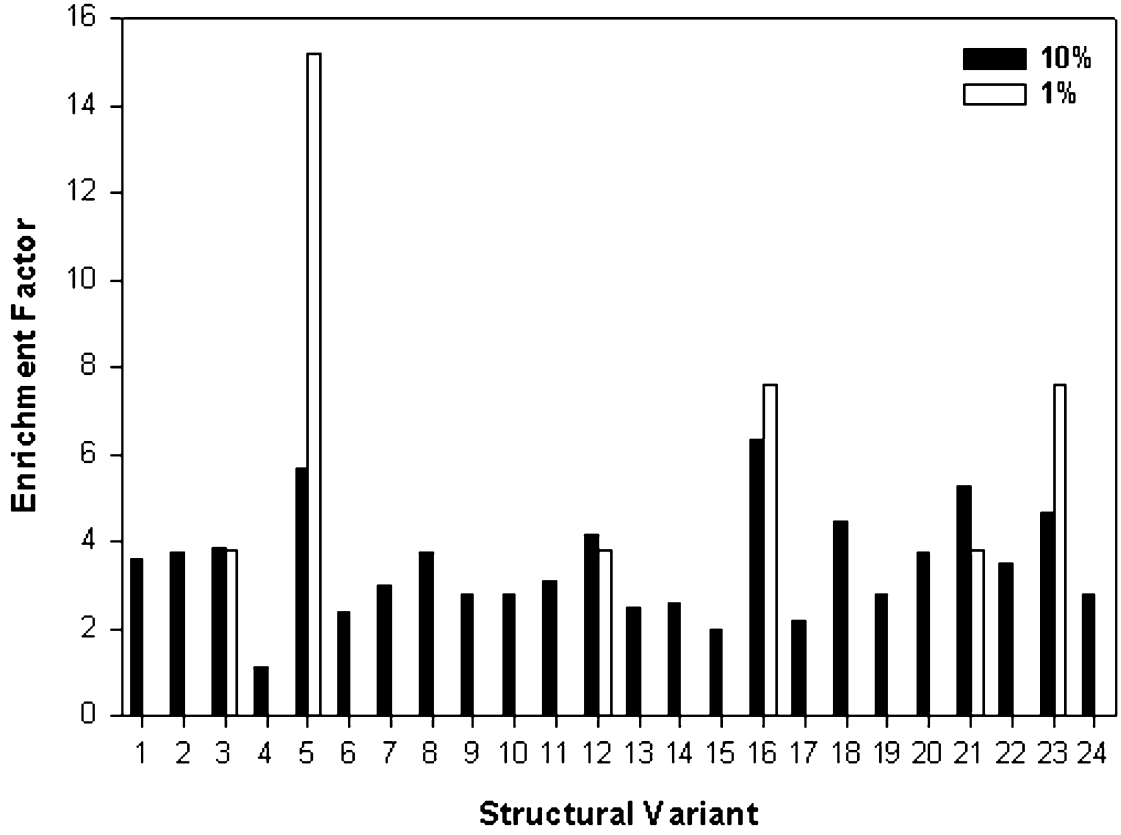
FIGURE 4. **CAT assay of selected phenothiazine derivatives.** (-), untreated cells, negative control; (+) 1.0 nM DHT, positive control; filled bars represent experiments where 1.0 nM DHT was added together with ligand at indicated concentration; white bars represent experiments where ligand alone was added at indicated concentration. Refer to Table 2 for ligand structures. **A.** D4; **B.** flutamide.

FIGURE 5. **Nuclear translocation experiments. AR**, untreated cells (negative control); **DAPI**, 4,6-diamidino-2-phenylindole; overlay column shows superimposition of untreated cells with cells treated with DHT (1.0 μM), indicated ligand (1.0 μ) or DHT/ligand. Refer to Table 2 for ligand structures.

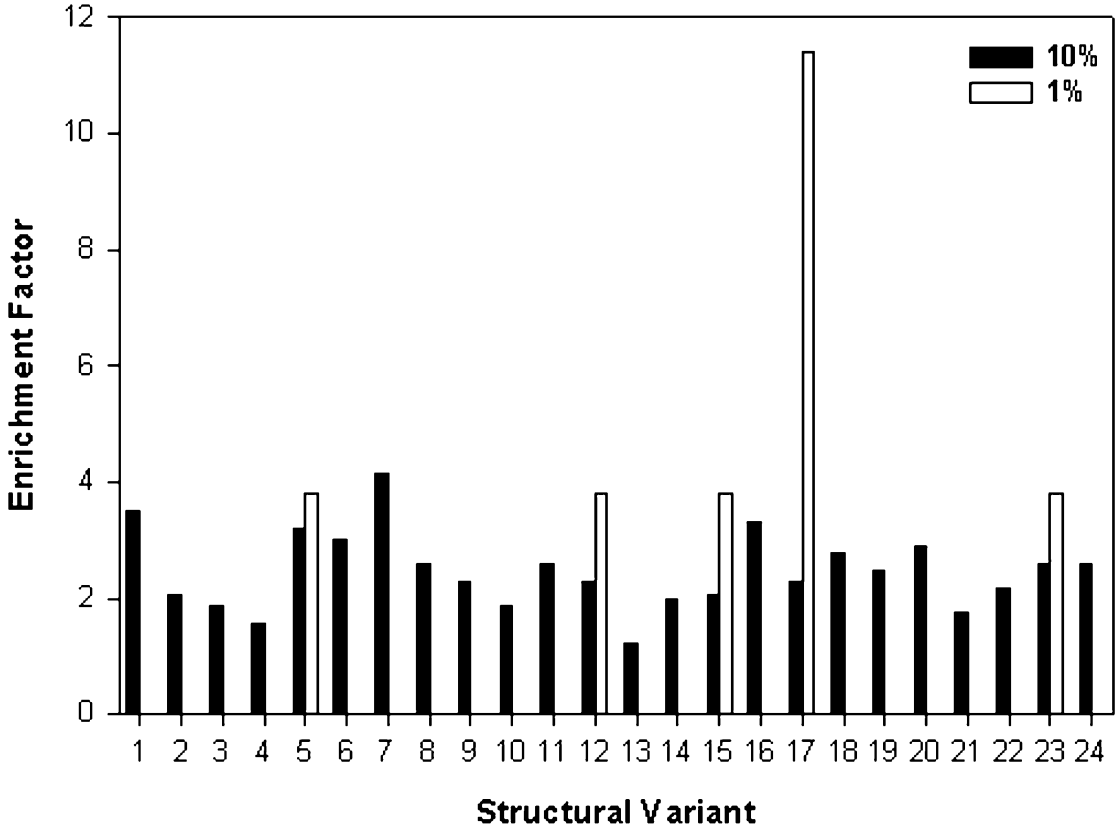
FIGURE 6. **The D4 ligand PSA Assay.** (-), raw cells (negative control); (+), 50.0 nM DHT alone (positive control). The ligand was added to the cells at indicated concentrations together with 50.0 nM DHT. The films were digitized, and PSA expression data were normalized by α-tubulin content. **Inset:** Typical Western blot; PSA – prostate specific antigen, TUB - α-tubulin.

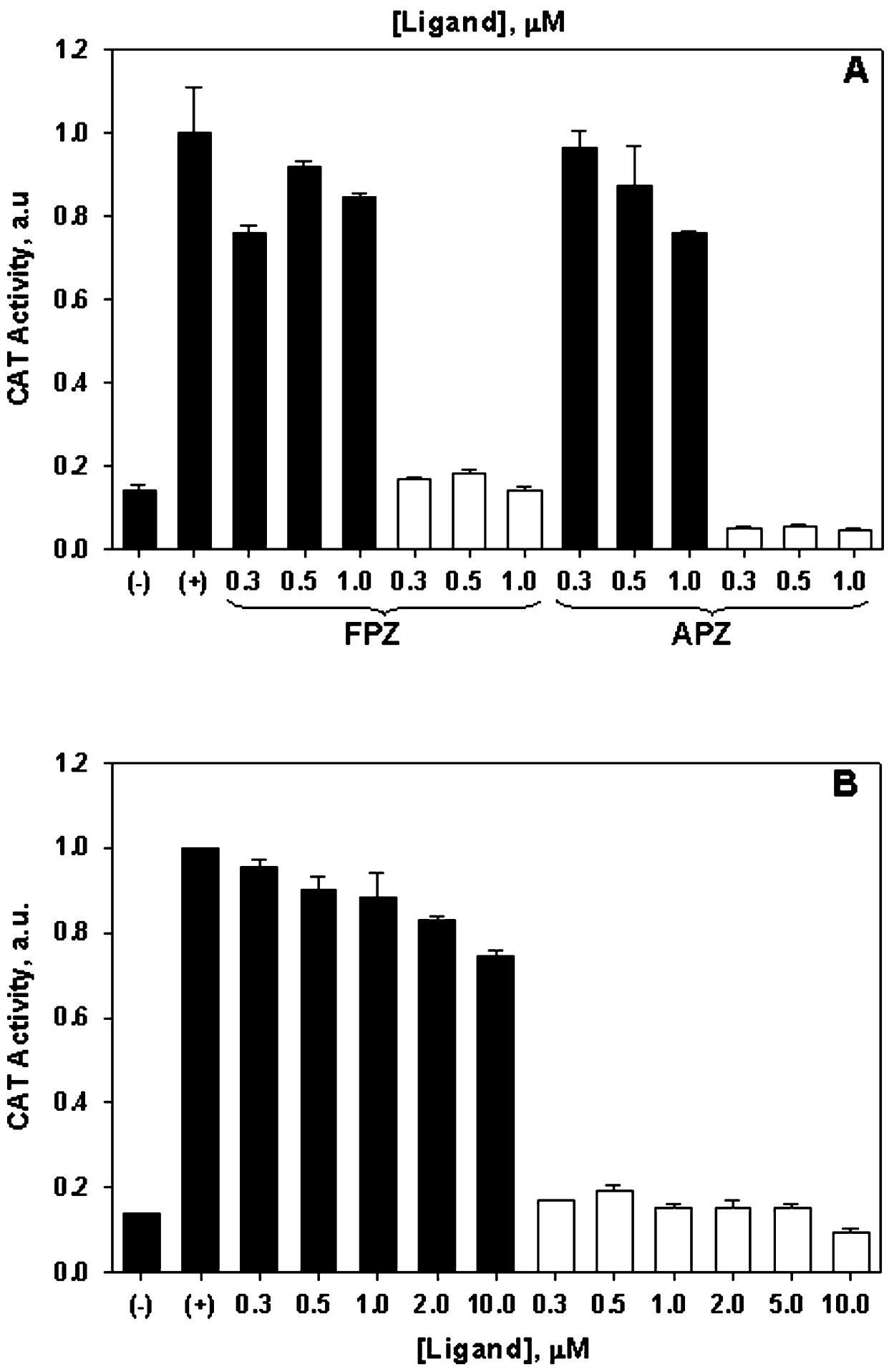

Model A
Dock Database A
Model B
Refine receptor side chains for each ligand in its most probable binding mode
24 derivatives of Model A
24 derivatives of Model B
• Dock Database C
• Determine docking score threshold value retaining 1% and 10% of top binders (50 and 500 compounds, respectively)
• Dock Database B
• Apply 1% and 10% docking score thresholds
• Select models with highest enrichment factors
Optimized Model A
Optimized Model B

Model A



Model B

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CAT Activity, a.u.
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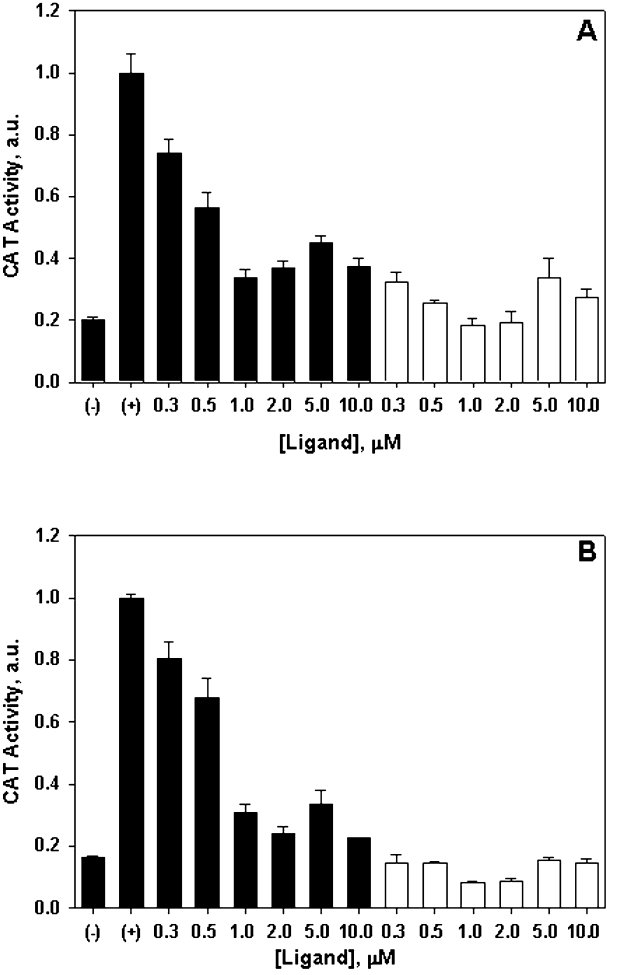
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CAT Activity, a.u.
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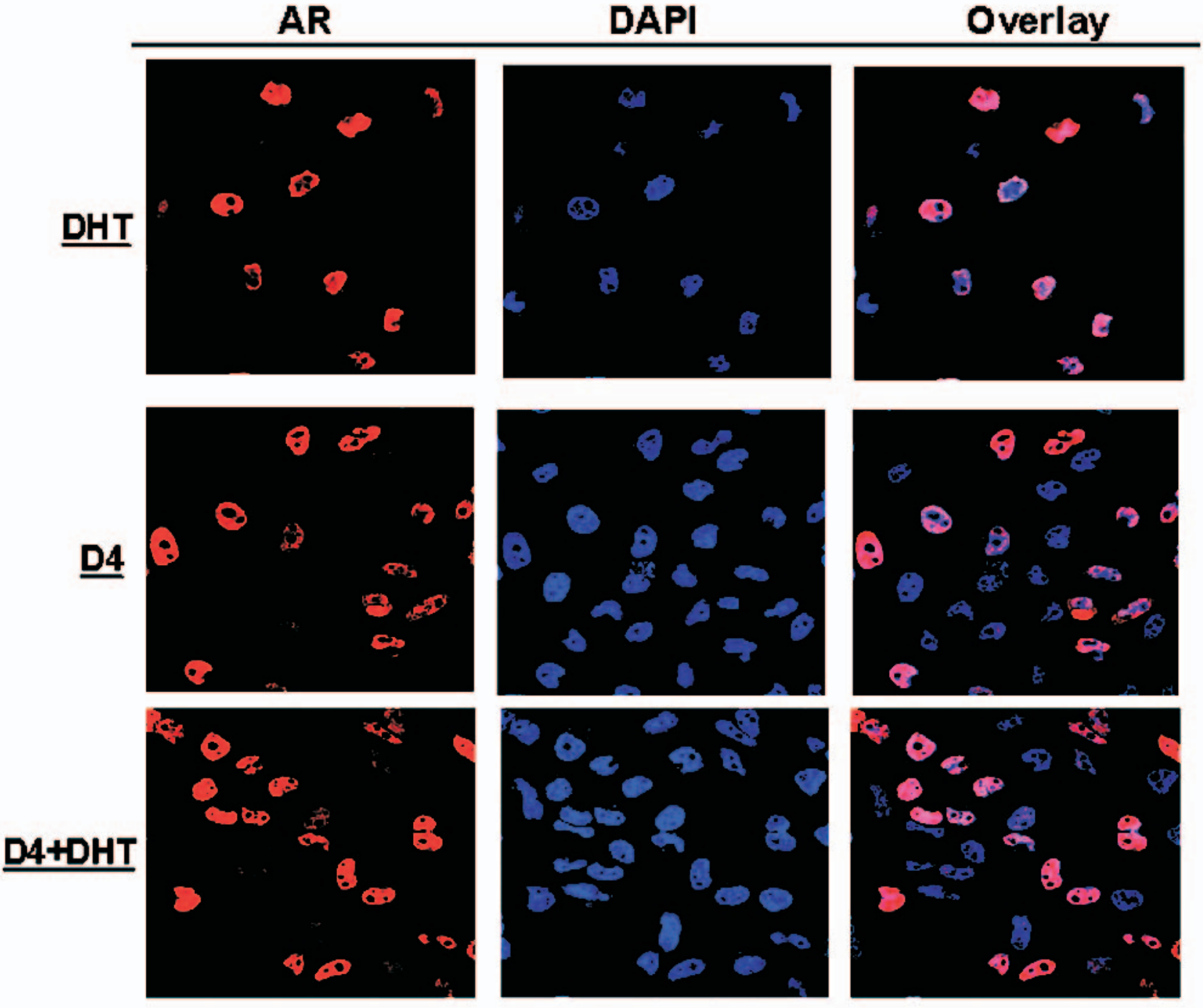
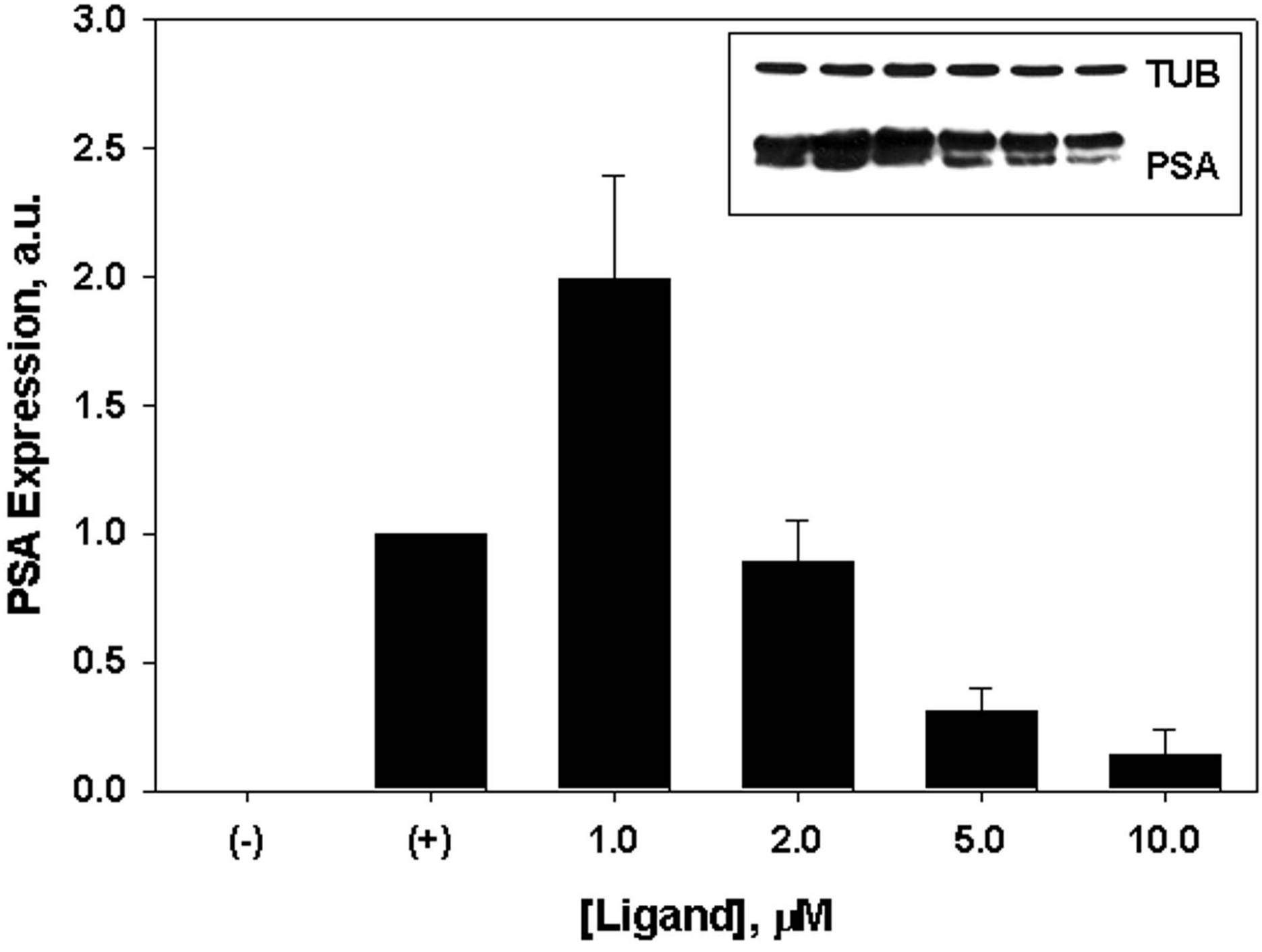

AR
DAPI
Overlay
DHT
D4
D4+DHT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PSA Expression, a.u.
TUB
PSA
(-)
(+)
1.0
2.0
5.0
10.0
[Ligand], μM

TABLE 1. **Selected marketed oral drugs listed with their name, pharmaceutical class and clinical application.**

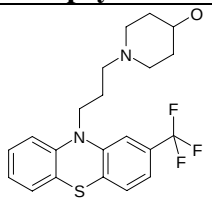
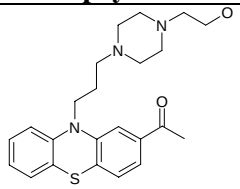
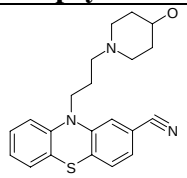
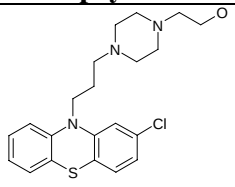
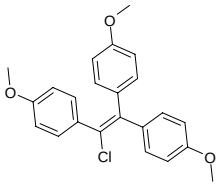
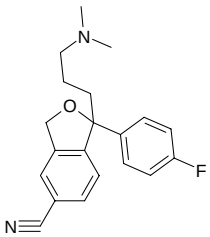
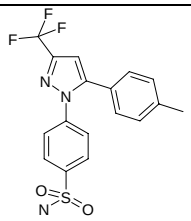
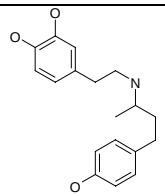
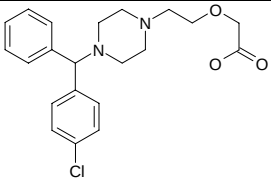
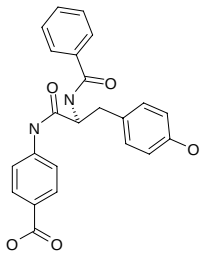
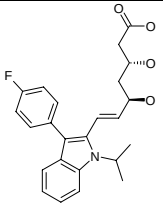
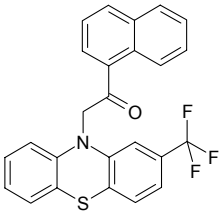
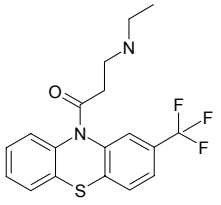
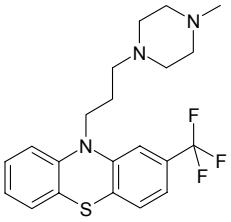
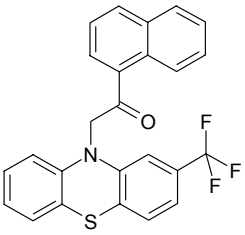
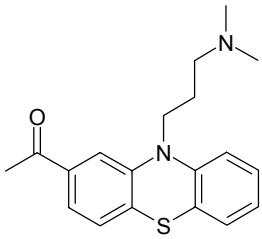
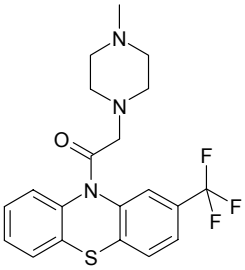
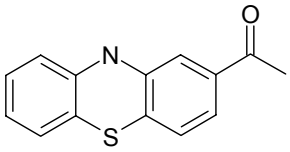
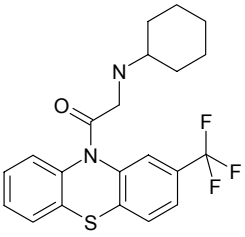
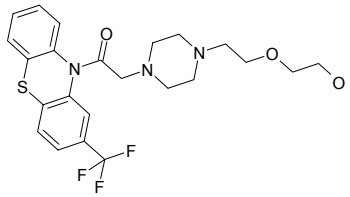
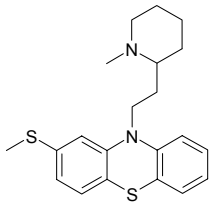
Fluphenazine **Phenothiazine, antipsychotic**	**Acetophenazine** **Phenothiazine, antipsychotic**	**Periciazine** **Phenothiazine, antipsychotic**	**Perphenazine** **Phenothiazine, antipsychotic**
			
Chlorotrianisene **Estrogen, menopause treatment**	**Citalopram** **DHT reuptake inhibitor, antidepressive**	**Celecoxib** **Nonsteroidal AID, arthritis symptoms**	**Dobutamine** **Adrenergic β-agonist, cardiac anomalies**
			
Cetirizine **Antihistamine, Allergy symptoms**	**Bentiromide** **Diagnostic agent, pancreatic function**	**Fluvastatin** **reductase inhibitor, anticholesterol**	
			

TABLE 2. **Antiandrogen activity of phenothiazine derivatives as determined by CAT assay.**

D1	**D2**	**D3**	**D4**
			
AR(-) = 9%*	**AR(-) = 2%**	**AR(-) =3%**	**AR(-) = 47%**
D5	***D6***	***D7***	***D8***
			
AR(-) = n/a	**AR(-) = n/a**	**AR(-) = 12%**	**AR(-) = n/a**
D9	***D10***		
			
AR(-) = n/a	**AR(-) = 2%**		

*antiandrogen activity is expressed as degree of inhibition of the reporter gene transactivation by 500 nM of indicated ligand in the presence of 1.0 nM DHT.